Effect of the tachykinin NK₁ receptor antagonists, RP 67580 and SR 140333, on electrically-evoked substance P release from rat spinal cord

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1 The effects of the non-peptide tachykinin NK₁ receptor antagonists, RP 67580, SR 140333, CP-96,345 and CP-99,994 have been investigated on electrically-evoked release of substance P-like immunoreactivity (SP-LI) from rat spinal cord slices.

2 RP 67580 (10 nM) and SR 140333 (1 nM), perfused 5 min prior to and during 8 min stimulation of the dorsal roots (20 V, 0.5 ms, 1 Hz), significantly enhanced SP-LI release by 213 ± 43 ($n = 8$) and 203 ± 31 ($n = 5$) % of control evoked release ($187 \pm 16\%$ of basal outflow, $n = 22$) respectively. Neither compound modified basal outflow of SP-LI (15.3 ± 2.5 fmol/8 ml, $n = 10$).

3 RP 67580 (10 nM) did not modify electrically-evoked release of calcitonin gene-related peptide-LI from rat spinal cord slices.

4 CP-96,345 (10 nM) and CP-99,994 (1 and 10 nM) did not alter electrically-evoked SP-LI release; however, they both inhibited release at $1 \mu\text{M}$. Inhibition was also induced by $1 \mu\text{M}$ RP 67580 but not $1 \mu\text{M}$ SR 140333.

5 The effect of the NK₁ receptor agonists, [Sar⁹Met (O₂)¹¹]SP and [Sar⁹]SP, could not be tested on SP-LI release due to interference with the substance P radioimmunoassay (RIA). The other NK₁ receptor agonists used, GR 73632, [Pro⁹]SP and septide, which did not interfere with the RIA, increased SP-LI basal outflow by $1807 \pm 713\%$ ($n = 3$), $1259 \pm 160\%$ ($n = 3$) and $620 \pm 69\%$ ($n = 3$) at 10 nM, 1 nM and $1 \mu\text{M}$, respectively. At the same concentrations, the three agonists also enhanced electrically-evoked SP-LI release by $204 \pm 38\%$ ($n = 6$), $753 \pm 40\%$ ($n = 3$) and $504 \pm 97\%$ ($n = 3$), respectively. The GR 73632 (10 nM)-induced increase in electrically-evoked SP-LI release, was not prevented by SR 140333 (100 nM). None of the agonists inhibited SP-LI release at lower concentrations (0.1 nM GR 73632; 0.01 and 0.1 nM [Pro⁹]SP and 1–100 nM septide).

6 NKA and NKB, at concentrations up to 10 nM which did not interfere with the RIA, did not modify electrically-evoked release of SP-LI.

7 The ability of NK₁ receptor antagonists to enhance electrically-evoked SP-LI release supports the concept of an NK₁ autoreceptor control mechanism at substance P nerve terminals within the dorsal horn of the rat spinal cord.

Keywords: SR 140333; RP 67580; substance P; NK₁ receptor; autoreceptor; spinal cord

Introduction

The undecapeptide substance P (SP) is a member of the tachykinin family of peptides. It is widely distributed throughout the mammalian central nervous system (CNS), including the spinal cord where it is highly localized in the superficial layers (Hökfelt *et al.*, 1975). In particular, SP has been identified in small diameter fibres within the dorsal roots of the rat spinal cord that have been shown to terminate within laminae I and II of the dorsal horn (Tuchscherer & Seybold, 1985). Other peptides such as calcitonin gene-related peptide (CGRP) (Wiesenfeld-Hallin *et al.*, 1984), galanin (Skofitsch & Jacobowitz, 1985), somatostatin (Hökfelt *et al.*, 1976), neurokinin A (NKA) (Kanazawa *et al.*, 1984) and dicarboxylic acids like glutamate and D-aspartate (Potashner & Tran, 1984) are also present in mammalian primary afferent terminals. SP appears to coexist with CGRP, galanin (Tuchscherer & Seybold, 1989) and glutamate (Biasi & Rustioni, 1988) in varicosities within the dorsal horn. It is possible that these co-localized agents may interact in the dorsal horn, at both presynaptic and postsynaptic sites, possibly to regulate sensory information transfer at the first CNS synaptic relay (Rusin *et al.*, 1992). For example, it is known that tachykinins and CGRP can increase glutamate release from primary afferent terminals with both peptides inducing a calcium-independent increase of either basal or electrically-evoked release of glutamate *in vitro* (Kawagoe *et al.*, 1986; Kangrga *et al.*, 1990; Kangrga &

Randić, 1990). SP has also been reported to increase glutamate release *in vivo* (Smullin *et al.*, 1990). A possible interaction between the peptides contained in primary afferent terminals has also been investigated and CGRP has been reported to increase SP release partly through inhibition of its degradation (Le Greves *et al.*, 1985; Oku *et al.*, 1987). However, no information is available on possible modulation by each peptide of its own release from primary afferent terminals.

We have recently reported that electrical stimulation of the dorsal roots induces a significant and consistent release of endogenous SP from lumbo-sacral horizontal slices of rat spinal cord (Malcangio & Bowery, 1993). SP release was calcium-dependent, tetrodotoxin-sensitive and inhibited by the amino acid neurotransmitter, γ -aminobutyric acid (GABA) through GABA_B receptors (Malcangio & Bowery, 1993). The availability of an experimental system which readily allows the assessment of endogenous SP release, has prompted us to investigate the possible existence of a SP autoreceptor on primary afferent terminals of the rat spinal cord. Tachykinin receptors are currently classified into three types referred to as NK₁, NK₂ and NK₃ (see review by Maggi *et al.*, 1993). Recently, selective and metabolically stable antagonists with high affinity for tachykinin receptors have been developed (see Watling, 1992) and, of these, four have been examined in the present study (Figure 1). CP-96,345

was the first non-peptide NK₁ receptor antagonist to be developed (Snider *et al.*, 1991) and shows higher affinity for NK₁ receptors in guinea-pig and human tissue (pK_i 9.6 ± 0.08 in human IM9 cells, McLean *et al.*, 1993) than in rat and mouse (Watling *et al.*, 1991; Gitter *et al.*, 1991; Beresford *et al.*, 1991; Barr & Watson, 1993). However, this NK₁ receptor antagonist also possesses non-specific effects such as interference with Ca²⁺ mobilization via an interaction with the L-type calcium channel, as shown by its ability to inhibit [³H]-diltiazem binding in rat cerebral cortex (IC_{50} 22.5 nM) (Schmidt *et al.*, 1992; Guard *et al.*, 1993), and depression of voltage-dependent sodium currents in neurones cultured from rat cortex (IC_{50} $18 \pm 2 \mu M$ at 0.1 Hz, Caesar *et al.*, 1993). A more recent derivative, CP 99,994, has similar affinity for NK₁ receptors in human IM9 cells (pK_i 9.6 ± 0.05), but considerably lower L-type calcium channel blocking activity (IC_{50} $3 \mu M$) (McLean *et al.*, 1993). Furthermore, this antagonist also has lower affinity for the rat caudate (pIC_{50} 6.21 ± 0.05) than for the human caudate/putamen NK₁ receptor (pIC_{50} 8.78 ± 0.07). RP 67580, is another selective non-peptide antagonist at the NK₁ receptor which has been reported to displace, competitively, [³H]-SP binding from rat brain membranes (K_i 4.1 nM) and to antagonize SP-induced contractions of the guinea-pig isolated ileum (pA_2 7.16) (Garret *et al.*, 1991). However, it shows the opposite species specificity to CP-96,345 being selective for the rat NK₁ receptor. Finally, SR 140333, has been reported to be a potent non-peptide NK₁ antagonist both in *in vivo* (Jung *et al.*, 1994) and *in vitro* models and shows no species selectivity (Emonds-Alt *et al.*, 1993).

In this study, we have compared the effects of RP 67580, CP-96,345, CP-99,994 and SR 140333 on electrically-evoked SP-LI release from rat isolated spinal cord. Agonists for tachykinin receptors were also employed (Figure 1) to investigate whether SP can regulate its own release from primary afferent fibres through an autoreceptor control mechanism.

One such selective NK₁ receptor agonist used in this study was septide ([pGlu⁶,L-Pro⁹]SP(6-11); Figure 1), a hexapeptide derivative of the SP C-terminal sequence (Laufer *et al.*, 1986), which shows higher affinity for the NK₁ binding sites in the guinea-pig ileum than for those in the rat brain (Lee *et al.*, 1986).

Methods

Rat spinal cord slice preparation and superfusion

Horizontal spinal cord slices were obtained from adult male Wistar rats (~250 g) as previously described (Malcangio & Bowery, 1993). Briefly, hemisected dorsal lumbosacral slices with attached dorsal roots were cut using a Vibratome (Campden Instruments, London) and mounted in a three-compartment chamber. The tissue was positioned in the central compartment where it was continuously perfused with Krebs solution at 1 ml min^{-1} and the dorsal roots were placed across two pairs of bipolar electrodes and immersed in mineral oil in the lateral compartments. Leak-proof partition barriers of perspex and paraffin grease ensured electrical isolation. After 1 h wash, normal Krebs solution (in mM: NaCl 118, KCl 4, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, CaCl₂ 2.5 and glucose, 11) was substituted with Krebs solution containing 0.1% bovine serum albumin, $100 \mu M$ captopril, $1 \mu M$ phosphoramidon, $20 \mu g \text{ ml}^{-1}$ bacitracin and dithiothreitol ($6 \mu M$) to prevent metabolic breakdown of SP. Eight-minute fractions were collected in the following order: 3 fractions to measure basal outflow, one fraction to evaluate release following electrical stimulation of 20 V, 0.5 ms, 1 Hz (SP) or 10 Hz (CGRP) either in the absence or presence of drugs and 3 fractions to evaluate recovery to basal levels. It is worth noting that a higher stimulation frequency was necessary to release consistent and significant amounts of

Figure 1 Structure of NK₁ receptor agonists and antagonists used in this study.

CGRP-LI. At the completion of each experiment, the slice was blotted, weighed and extracted. Samples (8 ml volume) were desalted by using 100 mg SEP-PAK C₁₈ reverse phase silica gel cartridges (Millipore) as already described for SP (Malcangio & Bowery, 1993). In the case of CGRP, cartridges were treated with 3 ml methanol followed by 6 ml trifluoroacetic acid 0.1% (TFA) and NaCl (3.5 g l⁻¹). Samples were then loaded into the cartridge followed by 6 ml TFA:NaCl and 4 ml TFA:methanol (6:4). CGRP was then eluted by using 2 ml methanol. The eluates were dried by evaporation at 55°C under nitrogen and stored at -80°C until they could be assayed for SP-LI or CGRP-LI content by RIA using the scintillation proximity assay bead technique (Amersham International, UK) (see Malcangio & Bowery, 1993).

SP and CGRP radioimmunoassays (RIA)

In each experiment, RIA standard curves were constructed for SP or CGRP by using known amounts of synthetic peptides ranging between 1 and 100 fmol per tube. The dried samples were resuspended in 300 µl of 50 mM phosphate buffer (pH 7.4) containing bovine serum albumin and 10 mM ethylenediaminetetracetic acid (EDTA) and assayed in amounts of SP immunoreactivity sufficient to place them within the range of the standard curve. SP or CGRP content were measured by using a polyclonal rabbit antiserum specific for the whole SP undecapeptide (Amersham International, UK) or for human CGRP (Peninsula Laboratories, U.S.A.). The incubation mixtures of each assay contained sample or standard (100 µl), tracer containing ~6000 c.p.m. of the isotope (100 µl), diluted antiserum (100 µl) and 50 µl of scintillation proximity assay protein A reagent. All samples were assayed in duplicate. The reaction mixture was vortexed and incubated at room temperature on a shaker for 48 h before being placed in the scintillation-counter.

Most of the compounds used in this study were checked for possible interference with the specific RIA for SP and only concentrations below the assay sensitivity (1 fmol per tube) were employed (Table 1).

Drugs

GR 73632 (δ-Ava-Phe-Phe-Pro-MeLeu-Met-NH₂) was a kind gift of Dr C.C. Jordan, Glaxo Group Research, Hertfordshire, U.K. Peptides were purchased from the following sources: NKA, NKB, [Sar⁹]SP, [Sar⁹Met(O₂)¹¹]SP, [Pro⁹]SP and septide (Neosystem, Strasbourg, France/SNPE Croydon, U.K.); SP and CGRP (Sigma), CP-96,345 ([2(S,3S)-*cis*-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]] octan-3-amine and CP 99,994 (+)-(2S,3S)-3-methoxybenzylamino-2-phenylpiperidine were a gift of Dr Kadin, Pfizer Inc, Groton, U.S.A.; RP 67580 ([3aR,7aR]-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)-ethyl] perhydroisoindol-4-one) and RP 68651 ([3aS,7aS]-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)-ethyl]perhydroisoindol-4-one) were kindly provided by Dr C. Garret, Rhône-Poulenc Rorer, Vitry, France; SR 140333 ((S)-1-[2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)-piperidin-3-yl]ethyl]-4-phenyl-1-azoniabicyclo[2.2.2] octane chloride) was kindly supplied by Dr X. Emonds-Alt, Sanofi Recherche, Montpellier, France. [¹²⁵I]-Bolton Hunter SP, [¹²⁵I]-CGRP and rabbit antiserum raised against SP (Amersham International, UK), rabbit antiserum against CGRP (Peninsula Laboratories, U.S.A.)

[Sar⁹]SP, [Sar⁹Met(O₂)¹¹]SP, NKA and NKB were dissolved in dimethylsulphoxide in stock solutions (1 mM) and stored at -20°C. SP was dissolved in 0.1% acetic acid and stored at -80°C; [Pro⁹]SP, GR 73632 and CGRP were dissolved in water and stored at -80°C; septide was dissolved in acetic acid 11% and stored at -80°C. NK₁ receptor antagonists

were dissolved in distilled water just before use except for RP 67580 which was previously dissolved in 0.1 M HCl.

Statistical analysis and calculation

Values were calculated as fmol SP-LI released by electrical stimulation after subtraction of basal outflow. Statistical analysis of the data was performed using a non-parametric test (Mann-Whitney U test). Values for Figure 3 and Tables 2 and 4 were calculated according to the formula:

$$100 \times \frac{\text{fmol evoked (NK}_1 \text{ receptor agonist or antagonist)} - \text{fmol (basal)}}{\text{fmol evoked (control)} - \text{fmol (basal)}}$$

Results

Electrical stimulation (20 V, 0.5 ms, 1 Hz) of the dorsal roots for 8 min induced a significant release (210 ± 0.1 fmol µg⁻¹ tissue, $n = 10$ $P < 0.01$) of substance P-like immunoreactivity (SP-LI) from rat spinal cord slices, which was approximately twice the basal level (90 ± 0.01 fmol µg⁻¹ tissue). This evoked release was Ca²⁺-dependent and inhibited by tetrodotoxin (1 µM) (Malcangio & Bowery, 1993) (data not shown).

In an attempt to determine whether the amount released

Table 1 Cross-reactivity between substance P (SP), neurokinin A (NKA), NKB and NK₁ receptor agonists and antagonists in the specific radioimmunoassay (RIA) for SP

Drug	Amount added	Apparent amount detected as SP
[Sar ⁹]SP	1-100 fmol	1-100 fmol
[Sar ⁹ Met(O ₂) ¹¹]SP	1-100 fmol	1-100 fmol
[Pro ⁹]SP	1-100 pmol	0-75 pmol
Septide	1-100 pmol	0-3 pmol
GR 73632	1-100 pmol	0-3.9 pmol
NKA	1-100 pmol	0 pmol
NKB	1-100 pmol	0 pmol
RP 67580	1-100 pmol	0 pmol

SP was used as standard (1-100 fmol per tube) for the RIA.

Table 2 Comparison between the effects of RP 67580, SR 140333, CP-96,345 and CP-99,994 on electrically-evoked substance P-like immunoreactivity (SP-LI) release from rat spinal cord slices

Drug	[nM]	n	SP-LI release (% of control evoked release)
RP 67580	1	3	94 ± 13
	3	3	170 ± 59
	10	8	213 ± 43*
	100	4	81 ± 8.5
	1000	6	57 ± 9.8*
SR 140333	0.1	4	154 ± 37
	1	5	203 ± 31*
	10	3	72 ± 25
	100	3	107 ± 36.3
CP-96,345	10	3	92.6 ± 22.4
	1000	3	26.4 ± 7.0*
CP-99,994	1	3	106.8 ± 30.8
	10	3	124 ± 41.7
	100	3	61.6 ± 3.17
	1000	5	50 ± 10.4*

Drugs were perfused 5 min prior to and during 8 min stimulation (20 V, 0.5 ms, 1 Hz). In control slices, electrically-evoked release - basal outflow was 16.8 ± 2.7 fmol per fraction ($n = 22$).

* $P < 0.05$, Mann-Whitney U test.

by stimulation was limited by possible feedback inhibition, the tissue was superfused with the NK₁ receptor antagonist, RP 67580, at concentrations between 1–1000 nM. RP 67580 (10 nM) significantly increased the amount of SP-LI released (Figure 2). This effect was concentration-dependent and also stereospecific since the inactive isomer, RP 68651, was without effect at 10 nM and 100 nM (Figure 3). The concentration-response curve to RP 67580 appeared to be 'bell-shaped' in that concentrations above 10 nM failed to enhance the evoked release of SP-LI. In fact, at 1 µM, a significant inhibition of the evoked release was observed (Figure 3).

The NK₁ receptor antagonist, SR 140333, also induced a significant increase in the electrically-evoked release of SP-LI at concentrations even lower (1 nM) than for RP 67580 (Table 2). However, at no concentration was any inhibition of release detected. The NK₁ receptor antagonist, CP-96,345,

Figure 2 Effect of RP 67580 10 nM (● *n* = 3) on substance P-like immunoreactivity (SP-LI) release from rat spinal cord slices induced by electrical stimulation of the dorsal roots for 8 min (solid horizontal bar) as compared to control slices (○, *n* = 3). Drug was perfused 5 min prior to and during stimulation (hatched horizontal bar). Stimulation parameters were as follows: 20 V, 0.5 ms, 1 Hz. **P* < 0.05 Mann-Whitney U test.

Figure 3 Concentration-dependent increase of electrically-evoked release of substance P-like immunoreactivity (SP-LI) from rat spinal cord slices induced by RP 67580 (●), and lack of effect of RP 68651 (□). Values are mean ± s.e. mean of 4–8 experiments and are calculated according to the formula in the Methods section. Stimulation parameters and drug perfusion were as described in Figure 2. In the absence of drug, stimulated control SP-LI release was 27.5 ± 2.9 fmol per fraction including 15.0 ± 1.2 fmol per fraction of basal outflow, *n* = 9. **P* < 0.05 RP 67580 versus control, †*P* = 0.05 RP 67580 versus control, Mann-Whitney U test.

at a concentration of 10 nM, failed to modify the evoked release of SP-LI but, like RP 67580, it significantly inhibited release at 1 µM (Table 2). At this concentration and at 100 nM, CP-99,994 also inhibited SP-LI release, whereas it did not modify the release at 1 and 10 nM (Table 2). None of the NK₁ receptor antagonists altered basal outflow of SP-LI at any concentration tested (Table 3).

The enhancement of SP-LI release produced by RP 67580 appeared to be selective in that the evoked release of CGRP-LI was unaltered by the same concentration (10 nM) of the NK₁ receptor antagonist (Figure 4).

To characterize further the modulation of SP release in spinal cord, an attempt was made to investigate the effects of the selective NK₁ receptor agonists, [Sar⁹]SP, [Pro⁹]SP, [Sar⁹Met(O₂)¹¹]SP, septide and GR 73632. Unfortunately, [Sar⁹Met(O₂)¹¹]SP and [Sar⁹]SP interfered with the RIA even at low concentrations (1–100 fmol per tube, Table 1) and could not therefore be used. However, GR 73632, [Pro⁹]SP and septide did not appear to interfere with the RIA even at 50–100 pmol/tube and were therefore tested. GR 73632

Figure 4 Effect of RP 67580 10 nM (●) on calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) release from rat spinal cord induced by electrical stimulation of the dorsal roots for 8 min (solid horizontal bar) compared to control slices (○). Drug was present 5 min prior to and during stimulation (hatched horizontal bar). Stimulation parameters were as follows: 20 V, 0.5 ms, 10 Hz. Values are means ± s.e. mean of 3 experiments.

Table 3 Comparison of the effects of RP 67580, SR 140333, CP-96,345 and CP-99,994 on substance P-like immunoreactivity (SP-LI) basal outflow from rat spinal cord slices			
Drug	[nM]	<i>n</i>	SP-LI release (% of control evoked release)
RP 67580	1	3	89.4 ± 12.8
	3	3	102 ± 12.3
	10	8	133.0 ± 18.1
	100	4	135.0 ± 23.5
	1000	6	111.0 ± 14.5
SR 140333	0.1	4	96.6 ± 10.2
	1	5	111.0 ± 6.9
	10	3	100.0 ± 1.0
	100	3	110.0 ± 4.5
CP-96,345	10	3	100.6 ± 6.3
	1000	3	114.3 ± 15.5
CP-99,994	1	3	115.0 ± 4.0
	10	3	112.3 ± 4.3
	100	3	153.0 ± 48.4
	1000	5	126.0 ± 11.5

Drugs were present for 5 min out of 8 min collection period. Basal outflow of SP-LI was 15.3 ± 2.5 fmol per fraction (*n* = 10) in the presence of peptidase inhibitors.

(10 nM), [Pro⁹]SP (1 nM) and septide (1 μ M) significantly increased the basal outflow of SP-LI in the absence of peptidase inhibitors (Figure 5) and electrically-evoked SP-LI release (Table 4). GR 73632 (10 nM) also induced a significant increase in basal outflow in the presence of peptidase inhibitors (22 ± 5.6 fmol per fraction over basal, $n = 3$, $P < 0.05$) and enhanced electrically-evoked release of SP-LI (Figure 6a and b). However, neither the effects on basal nor electrically-evoked release could be antagonized by SR 140333 (100 nM) (Figure 6b). [Pro⁹]SP, perfused at a concentration of 0.01 nM which did not interfere with the RIA or increase SP-LI basal outflow, inhibited ($P = 0.05$) electrically-evoked SP-LI release (Table 4). Higher concentrations of [Pro⁹]SP (0.1 and 1 nM) appeared to enhance the release of SP-LI (Table 4) which could be attributed to the apparent increase in basal outflow (Figure 5). Septide, did not modify electrically-evoked SP-LI release in the concentration range 1–100 nM (Table 4). The tachykinins NKA and NKB could

Table 4 Comparison between the effects of GR 73632, [Pro⁹]SP and septide on electrically-evoked substance P-like immunoreactivity (SP-LI) release from rat spinal cord slices

Drug	[nM]	n	SP-LI release (% of control evoked release)
GR 73632	0.1	4	192 $\pm$ 67
	10	6	204 $\pm$ 38*
[Pro ⁹]SP	0.01	4	38 $\pm$ 12†
	0.1	2	180
	1	3	753 $\pm$ 40
Septide	1	3	121 $\pm$ 56
	10	3	97 $\pm$ 32
	100	4	219 $\pm$ 78
	1000	3	504 $\pm$ 97

Drugs were perfused 1 min prior to and during 8 min stimulation (20 V, 0.5 ms, 1 Hz). In control slices, electrically-evoked release – basal outflow was 19.5 ± 2.9 fmol per fraction ($n = 12$).

* $P < 0.05$, † $P = 0.05$, Mann-Whitney U test.

Figure 5 Effect of [Pro⁹]SP (1 nM), (▨) GR 73632 (10 nM), (◊) and septide (1 μ M) (■) on basal outflow of substance P-like immunoreactivity (SP-LI) detected in the absence of peptidase inhibitors. Abscissae indicate time at which 8 ml fractions were collected. Open columns represent SP-LI content in presence of peptidase inhibitors which was reduced significantly in the absence of peptidase inhibitors (solid columns). See methods for details of peptidase inhibitors used. $n = 9$ experiments for initial 5 columns and 3 experiments for final 3 columns. * $P < 0.05$, ** $P < 0.01$.

Figure 6 Effect of GR 73632 (10 nM, $n = 6$) (●) on substance P-like immunoreactivity (SP-LI) release from rat spinal cord slices induced by electrical stimulation of the dorsal roots for 8 min (solid horizontal bar). Control slices, $n = 4$ (○). Drug was present 1 min prior to and during stimulation (hatched horizontal bar) (a). Lack of antagonism of GR 73632 (10 nM)-induced increase in SP-LI release by SR 140333 (100 nM) (b). Stimulation parameters were as follows: 20 V, 0.5 ms, 1 Hz. Values are means $\pm$ s.e. mean of 3–5 experiments. * $P < 0.05$ Mann-Whitney U test.

be examined at concentrations up to 10 nM without interfering with the RIA. At this concentration, neither modified the electrically-evoked SP-LI release (104 ± 0.5 and $114.5 \pm 2.5\%$ of control evoked release, respectively, $n = 3$) or basal outflow (data not shown).

Discussion

In the present study, the specific antagonist for the rodent tachykinin NK₁ receptor, RP 67580, but not its inactive isomer RP 68651, dose-dependently increased the electrically-evoked release of SP-LI from rat spinal cord whilst producing no effect on basal SP-LI release. This effect was mimicked by the NK₁ receptor antagonist, SR 140333, which has similar or greater affinity for rat and human NK₁ receptors, but not by CP-99,994 and CP-96,345 which have low affinity for the rodent NK₁ receptor.

RP 67580- and SR 140333-induced increases in electrically-evoked SP-LI release support the concept of an autoreceptor control mechanism through which SP could modulate its own release from primary afferent terminals in the dorsal horn. It has already been suggested that SP, together with CGRP, may regulate glutamate and aspartate release from the same terminals (Kawagoe *et al.*, 1986; Kangrga *et al.*, 1990; Kangrga & Randić, 1990). On this basis, SP and CGRP autoreceptors have been proposed to exist on neuropeptide-containing primary afferent terminals (Levine *et al.*, 1993). Our data now provide evidence for this existence, thus providing a mechanism through which SP could exert a negative feed-back on its own release from primary afferent terminals since the NK₁ receptor antagonists, RP 67580 and

SR 140333, enhanced the release of SP-LI. Unfortunately, we could not demonstrate any inhibition of SP-LI release under our conditions since the available agonists all increased the apparent SP concentration in the perfusates, when used at concentrations reported to be effective in other studies (Petitet *et al.*, 1992; Meini *et al.*, 1994; Lee *et al.*, 1986; Hagan *et al.*, 1991) and this increase was not blocked by the NK₁ receptor antagonists.

RP 67580 did not modify CGRP-LI evoked release suggesting that, if there was any control exerted by endogenous SP on its release from primary afferent fibres, it could not be detected in our system.

The existence of an autoreceptor for SP on primary afferent terminals has previously been excluded on the basis of autoradiographic studies in which binding sites for SP increased in the dorsal horn of neonatally capsaicin-treated rats (Helke *et al.*, 1986). However, any loss of presynaptic sites could be missed by an up regulation of postsynaptic SP binding sites due to the removal of SP innervation and the number of presynaptic sites may be much lower than the postsynaptic levels. In addition, mismatch occurs for SP and its receptor at all levels of the brain and spinal cord and only when receptor subtypes are considered does there appear to be a better match with the peptide distribution (Herkenham, 1987).

The concentrations of RP 67580 (10 nM) and SR 14033 (1 nM) which enhanced the release of SP-LI through a putative presynaptic NK₁ receptor are in the range reported to be effective in *in vitro* models for postsynaptic NK₁ receptor activation (Garret *et al.*, 1991; Oury-Donat *et al.*, 1994). The present results provide no evidence to suggest that the putative autoreceptor and the postsynaptic receptor have different characteristics, but this is an important consideration requiring further study.

The lack of effect of RP 67580 and SR 140333 on basal SP-LI release suggests that there is no SP 'tone' at autoreceptors in the spinal cord, at least under our experimental conditions. The low basal level of SP-LI detected in our system is presumably too low to affect the autoreceptor and just reflects the small amount which is spontaneously released from stores within the tissue. The highest concentrations of RP 67580 and CP-96,345 used in this study inhibited the release of SP and this might be due to an interference with calcium entry into the primary afferent fibre terminals. In fact, it has recently been reported that racemic RP 67580 can inhibit [³H]-diltiazem binding to rabbit skeletal membranes (IC₅₀ 290 nM) and depresses high threshold calcium current in neurones cultured from rat cortex (10% inhibition at 10 µM) (Rupniak *et al.*, 1993). Since we know that the electrically-evoked release of SP in our preparation is Ca²⁺-dependent and can be blocked by tetrodotoxin (Malcangio & Bowery,

1993), it seems reasonable to assume that the inhibitory effect of higher concentrations of RP 67580 (1 µM) is unrelated to SP receptor antagonism, but more to non-specific effects on neurotransmission (Wang *et al.*, 1994). The Ca²⁺-blocking effect of RP 67580 is shared by the former non-peptide NK₁ receptor antagonist CP-96,345 (Schmidt *et al.*, 1992; Guard *et al.*, 1993). This compound has been shown to interact with L-type calcium channels in rat cerebral cortex membranes and furthermore to block voltage-dependent sodium currents in neurones cultured from rat cortex (Caesar *et al.*, 1993). This latter property might be more of a determinant for the block of SP release since the same effect was also induced by the same concentration of CP-99,994 which possesses less calcium channel-blocking activity than CP-96,345 (McLean *et al.*, 1993).

Experiments with the NK₁ receptor agonists, [Pro⁹]SP, GR 73632 and septide, revealed they could greatly increase the basal SP-LI content in the spinal cord perfusion, even at concentrations that did not cross-react with SP antiserum. The fact that they exerted this effect in the absence of peptidase inhibitors to a much greater extent than in their presence, may suggest a possible inhibition of the peptide breakdown. GR 73632 has been suggested to be a metabolically stable NK₁ receptor agonist (Hagan *et al.*, 1991), although this does not exclude the possibility that it itself might inhibit peptidase activity, thus reducing SP degradation.

Modulation of SP-LI release by activation of either NK₂ or NK₃ receptors is unlikely to be responsible since NKA and NKB did not modify SP-LI release. NKB is not contained in the primary afferent terminals (Ogawa *et al.*, 1985) whereas NKA is co-localized with SP-LI and has also been shown to increase glutamate release, from primary afferent terminals (Kangrga *et al.*, 1990). Its lack of effect on SP-LI release could be either attributed to the limited concentration we could use without interfering with the RIA or to an absence of NK₂-mediated modulation of SP-LI release from primary afferent terminals. This last possibility appears most likely in view of the fact that once released, NKA diffuses through the dorsal horn towards the deeper layers of the spinal cord (Duggan *et al.*, 1990), whereas SP remains localized in the substantia gelatinosa (Morton *et al.*, 1990).

In conclusion, we suggest the existence of an NK₁ autoreceptor for SP on primary afferent fibre terminals in the rat spinal cord. Whether this receptor is the same as that which is believed to regulate the release of coexisting transmitters (Levine *et al.*, 1993) remains to be determined.

This study was supported by grant B0196 from the Arthritis and Rheumatism Council.

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(Received January 28, 1994

Revised May 25, 1994

Accepted June 16, 1994)